

A CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF THE THREE BILE PIGMENTS IN SERUM

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In studying the aetiology of the different types of jaundice, much importance has been attached to the amount of "indirect" and "direct" bilirubin present in the blood. Cole, Lathe, and Billing (1954) have shown that in serum from patients with obstructive jaundice there are two bile pigments (pigment I and pigment II), which give a direct reaction in the van den Bergh test. Pigment I and pigment II can be separated from each other and from the indirect-reacting bile pigment, bilirubin, by reverse phase partition chromatography. Examination of the chromatograms has shown that there is considerable variation in the relative amounts of the three bile pigments. A quantitative method for their determination has therefore been developed so that a study of the clinical significance of the amounts of pigment I and pigment II can be made. The possibility that quicker and simpler methods for their determination might be employed has also been examined.

Methods

Apparatus.—It was not possible to elute the three bile pigments quantitatively from the usual type of partition chromatogram. A column (Fig. 1) was therefore designed so that, after separation of the three pigments, the supporting kieselguhr could be removed and divided into three portions, each of which contained a pigment. It was made from two 5-ml. syringes, the barrels of which were interchangeable. The hollow plungers were cut into three lengths (A, B, and C), the connecting surfaces were ground and a piece of glass tubing was fused to the top end (A). The nozzle of the syringe was removed from the barrel and annealed to portion C of the plunger. Portions A, B, and C were kept in position by the barrel. A length of fine-bore polythene tubing was fitted to the nozzle, which contained a small piece of cotton wool; this tubing was used to regulate the flow of solvents through the column by adjusting its height.

Solutions.—The following are required:—

- (1) Saturated ammonium sulphate solution.
- (2) Ethanol R.R.

(3) Solvent system: 50 ml. butanol; 45 ml. water; 5 ml. 0.05 M phosphate buffer, pH 6.0 (Cole, 1933).

(4) Freshly prepared alcoholic solution of diazotized sulphanilic acid (pH 4.1): 10 ml. 0.1% (w/v) sulphanilic acid in 0.25 N HCl; 0.3 ml. 0.5% (w/v) sodium nitrite; 20 ml. 1.43% (w/v) anhydrous disodium hydrogen phosphate; 90 ml. ethanol.

Procedure.—The chromatogram was prepared by stirring 1.5 g. silicone-treated kieselguhr (Howard and Martin, 1950) with 0.75 ml. of the upper phase of the butanol system and then gradually adding 10 ml. of the mobile aqueous phase until a smooth slurry had been made. The mixture was rapidly poured into the column and the excess fluid was drained off until the surface was just dry. The kieselguhr column held approximately 5 ml. mobile phase and was about 8.5 cm. long.

A protein-free solution of the pigments was obtained by adding to 1 ml. serum 0.18 ml. saturated ammonium sulphate solution and 2.5 ml. ethanol (Cole and Lathe, 1953). After standing in the dark for half an hour, the precipitate was removed by centrifugation and a sample of the supernatant solution, containing 50–100 μ g. total bile pigments, was taken to dryness *in vacuo* at room temperature. If the serum contained large amounts of bilirubin (which is not soluble in the mobile phase) then a small amount of the silicone-treated kieselguhr was added just before drying was complete together with 1 ml. ethanol; the bilirubin became adsorbed on to the kieselguhr and the solution was taken to dryness. During the drying procedure care was taken to ensure that there were no losses of pigment due to the fluid creeping up the side of the tube. If there are several specimens for analysis and the serum has a low lipid content, then the drying may be conveniently carried out in a vacuum desiccator containing calcium chloride.

The dried supernatant was transferred quantitatively to the top of the kieselguhr column using a total of not more than 0.5 ml. aqueous phase of the butanol-water solvent system. The pigmented solution was run into the kieselguhr and, when the top was almost dry, 4.5 ml. aqueous solution was added. The column was kept out of the sunlight and draughts and the solution permitted to flow through at a rate of not more than 1 ml. every 3 minutes, until the top of the kieselguhr column was

again almost dry and separation of the pigments had been achieved as shown in Fig. 1. The plunger was then slowly pushed through the barrel so that the three portions containing the pigments could be separated.

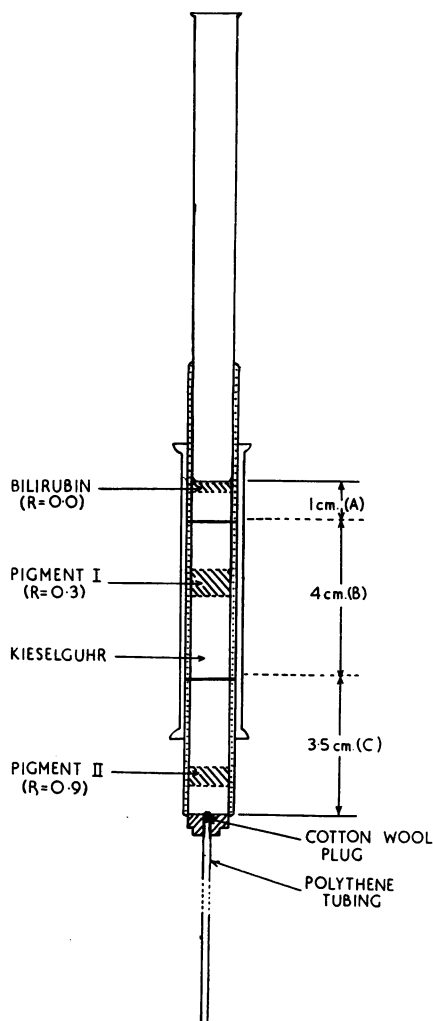


FIG. 1.—A microchromatographic apparatus made in three sections from the plungers of two 5-ml. syringes, which are kept in position by the syringe barrel. The three bands illustrate the relative positions of bilirubin, pigment I, and pigment II on the chromatogram.

The contents of each portion were transferred to glass-stoppered tubes. The pigments were converted to the corresponding azo-compound and extracted by shaking with 5–10 ml. alcoholic diazotized sulphanilic acid. After centrifugation the kieselguhr residue was re-extracted with 5 ml. ethanol. The solutions were combined for each pigment and the volumes measured. After 30 minutes readings were made in a Beckman

spectrophotometer at 525 $m\mu$ and the proportions of bilirubin, pigment I, and pigment II calculated. A methyl red standard (King and Coxon, 1950) was used.

Determinations of serum bile pigments were made using the method of Malloy and Evelyn (1937), readings for the "direct" bilirubin being taken after 30 minutes; "1 minute bilirubin" was determined as described by Ducci and Watson (1945).

Experimental

Reproducibility of Results.—Table I shows the results of five analyses carried out on one specimen of serum whose total bile pigment content was 20.8 mg./100 ml. serum. The coefficients of variation for bilirubin, pigment I, and pigment II, were 4.7%, 5.2%, and 5.2%, respectively. No attempt has been made to study the reproducibility of results at different bile pigment levels.

Chromatographic and Malloy-Evelyn Methods for "Direct Bilirubin" Compared.—The method of Malloy and Evelyn (1937) has been widely used in clinical circles to determine the amount of "direct bilirubin" in the serum of jaundiced patients. The results obtained from 27 sera using this method have been compared with those obtained chromatographically by adding the values for pigment I and pigment II (Fig. 2). Student's *t* test showed that there was no significant difference between the results obtained by the two methods.

Ten determinations of "direct bilirubin" were made by the Malloy and Evelyn method on the

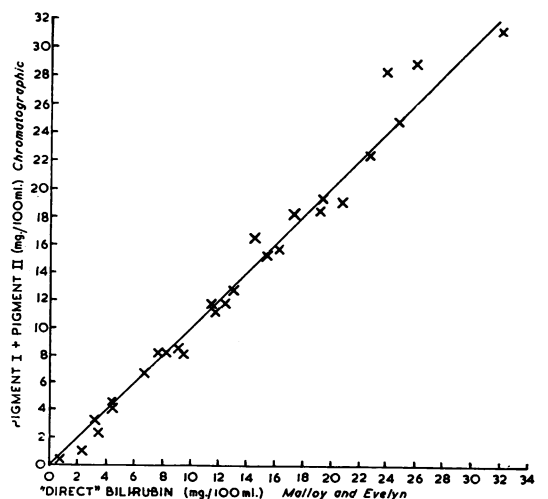


FIG. 2.—A comparison of the results for "direct bilirubin" obtained by the Malloy and Evelyn method and the chromatographic method.

specimen of serum used in the study on the reproducibility of results (Table I). A mean value of $16.75 \pm \text{S.D. } 0.41$ mg. per 100 ml. serum was

TABLE I
RESULTS OF FIVE ANALYSES ON ONE SPECIMEN OF SERUM

Specimen	Serum Bile Pigments (mg./100 ml.)			
	Bilirubin	Pigment I	Pigment II	Total Direct Pigment
1	4.23	8.84	7.53	16.37
2	4.04	9.75	7.26	17.01
3	4.25	9.79	6.60	16.39
4	3.81	10.18	6.88	17.06
5	3.98	9.42	7.26	16.68
Mean	4.06	9.59	7.11	16.70
Standard deviation	0.19	0.50	0.37	0.33
Coefficient of variation	4.7%	5.2%	5.2%	2.0%

obtained. This gave a coefficient of variation of 2.4% compared with 2% for the chromatographic method.

"1 Minute Bilirubin" and Pigment I.—Ducci and Watson (1945) have suggested that the diazotized bilirubin formed during the first minute of the direct diazo reaction in the Malloy and Evelyn method is due to the presence of a promptly reacting bilirubin, the remainder of the reaction being due to a delayed component. The possibility that this "1 minute bilirubin" could be identified

TABLE II
COMPARISON OF VALUES FOR PIGMENT I AND "1 MINUTE BILIRUBIN" IN 22 SAMPLES OF SERUM

Specimen No.	Pigment I (mg./100 ml. serum)	"1 Minute Bilirubin" (mg./100 ml. serum)	Pigment I—"1 Minute Bilirubin" $\times 100$ (%)
1	0.3	0.4	-33
2*	1.1	3.5	-218
3*	1.6	3.5	-119
4*	2.5	5.1	-104
5*	2.8	6.4	-129
6*	3.3	6.6	-100
7*	4.8	7.8	-63
8	5.2	6.4	+23
9*	5.5	12.7	-131
10	5.7	5.0	+14
11	5.7	5.1	+11
12	7.3	7.3	0
13	8.5	8.8	-4
14	10.3	13.0	-26
15	10.3	8.6	+17
16	10.4	10.8	-4
17	11.0	7.6	+31
18	13.3	17.5	-32
19	13.8	11.4	+17
20	21.2	16.2	+24
21	21.4	15.7	+27
22	22.6	20.4	+10
Mean	8.57	9.08	
Standard deviation	6.44	5.74	

* Specimen contained more pigment II than pigment I.

with either pigment I or pigment II has been investigated in the hopes of providing a quick method for their determination. Table II shows a comparison of the results obtained for pigment I and "1 minute bilirubin" determinations made on 22 samples of serum. Some similarity in the results was obtained when the serum contained pigment I as the dominant bile pigment, but none was obtained when pigment II was present in larger amounts than pigment I. Fig. 3 indicates that the amount of "1

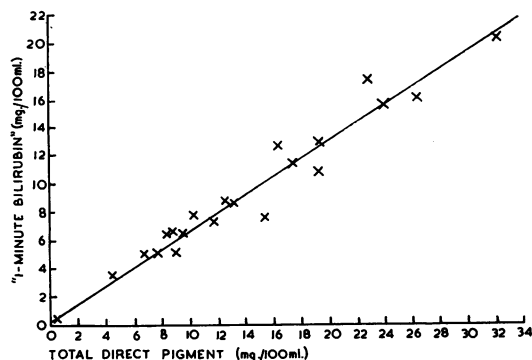


FIG. 3.—The relation between "direct bilirubin" and "1 minute bilirubin." Regression line $y = 0.64x + 0.24$ is superimposed.

minute bilirubin" present was more closely related to the total amount of direct-reacting pigment than to a particular pigment, a finding that agrees with that of Klatskin and Drill (1950).

Discussion

The instability of the direct-reacting bile pigments in serum is well known so that with any method for their quantitative determination considerable care must be taken to prevent their decomposition. The possibility of eluting both of these pigments from partition chromatograms was initially considered, but did not prove practicable since tailing of pigment I made the procedure too slow and elution of the pigment was not complete.

The use of small split columns enables the procedure to be carried out using about 1 ml. serum and has the advantage that the actual separation of the pigments takes less than 20 minutes. Provided excessive amounts of pigment are not used good separation can be achieved, and recoveries of 85 to 95% of the total pigment in the protein-free filtrate of the serum are obtained. Recovery experiments suggest that any losses that are incurred usually refer to the pigments as a whole and not to any particular pigment. The method is not easily applicable to sera whose total bile pigment concentration is less than 5 mg./100 ml. since

interference by lipids limits the amount of dried serum filtrate that can be run on a partition chromatogram.

The pigment adhering to an alcoholic precipitate of serum is known to be of the directly reacting type. The values given for bilirubin in serum, therefore, probably err on the high side. The accuracy of the chromatographic method is not greater than 5% so that it was agreeable to find that the values for the directly reacting pigments in serum by this method gave such good agreement with those determined by the method of Malloy and Evelyn, which involves no protein precipitation.

It was not possible to associate the prompt reacting "1 minute bilirubin" described by Ducci and Watson with either pigment I or pigment II, so that if information regarding the amounts of the two pigments is required the method described may be of use. If, however, only the total amount of direct-reacting pigments is wanted then the established and quicker method of Malloy and Evelyn will give equally reliable results.

Summary

"Bilirubin" as determined by the usual diazotization procedures consists of three related substances: bilirubin, and two pigments, which

give a direct reaction in the van den Bergh test (pigment I and pigment II). These three bile pigments can be separated by reverse phase partition chromatography. A quantitative method for their determination is described.

The Malloy and Evelyn (1937) method for determining "direct bilirubin" in serum is shown to give a reliable estimate of the total amount of pigment I and pigment II present.

The promptly reacting "1 minute bilirubin" described by Ducci and Watson (1945) could not be identified with either of the directly reacting bile pigments in serum.

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